

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problems Mailbox.**

STIC-ILL

NPL, GR180.J6  
418488

From: Holleran, Anne  
Sent: Wednesday, October 30, 2002 3:48 PM  
To: STIC-ILL  
Subject: refs. for 09/835,759

Please send copies of the following papers:

1. Keith, Cancer Immunology, Immunotherapy (2002) 51(10): 521-531
2. Mian Immunology and Medicine Series (2001) 30(Cancer Immunolgy ), 1-26
3. Timmerman J. Immunology (2000) 164(9): 4797-4803
4. Santin J. Virology (2000) 74(10): 4729-4737
5. Tanigawa J. Immunotherapy (2000) 23(5): 528-535
6. Flieger Hybridoma (1999) 18(1): 63-68
7. Sandmaier J. Immunotherapy (1999) 22(1): 54-66
8. Reinartz Hybridoma (1999) 18(1): 41-45
9. Lofthouse Vaccine (1997) 15(14): 1586-1593
10. Haagen Leukemia and Lymphoma (1995) 19(5-6): 381-393
11. Reddish Onkologie 91995) 18(suppl 1): 33-35
12. Apostolopoulos Cancer Res. (1994) 54(19): 5186-5193

Anne Holleran  
AU: 1642  
Tel: 308-8892  
RM: 8e03

mailbox: 8e12

# Linkage of Foreign Carrier Protein to a Self-Tumor Antigen Enhances the Immunogenicity of a Pulsed Dendritic Cell Vaccine<sup>1</sup>

John M. Timmerman and Ronald Levy<sup>2</sup>

The unique Ag-presenting capabilities of dendritic cells (DCs) make them attractive vehicles for the delivery of therapeutic cancer vaccines. While tumor Ag-pulsed DC vaccination has shown promising results in a variety of murine tumor models and early clinical trials, the optimal form of tumor Ag for use in DC pulsing has not been determined. We have studied DC vaccination using alternative forms of a soluble protein tumor Ag, the tumor-specific Ig idiotype (Id) expressed by a murine B cell lymphoma. Vaccination of mice with Id-pulsed DCs was able to induce anti-Id Abs only when the Id was modified to constitute a hapten-carrier system. DCs pulsed with Id proteins modified to include foreign constant regions, foreign constant regions plus GM-CSF, or linkage to keyhole limpet hemocyanin (KLH) carrier protein were increasingly potent in their ability to elicit anti-Id Abs. Vaccination with Id-KLH-pulsed DCs induced tumor-protective immunity superior to that obtained with Id-KLH plus a chemical adjuvant, and protection was not dependent upon effector T cells. Rather, protection was associated with the induction of high titers of anti-Id Abs of the IgG2a subclass, characteristic of a Th1 response. These findings have implications for the design of therapeutic Ag-pulsed DC vaccines for cancer immunotherapy in humans. *The Journal of Immunology*, 2000, 164: 4797–4803.

The pivotal role played by dendritic cells (DCs)<sup>3</sup> in the initiation of immune responses makes them an attractive cellular adjuvant for use in cancer vaccines (1). When pulsed with tumor-derived peptides or proteins or when transduced with tumor Ag-encoding viruses or nucleic acids and administered as a cellular vaccine, DCs have been shown to promote protective and even therapeutic anti-tumor immunity in a number of murine tumor models (2–12). These studies have formed the basis for the clinical use of DCs in active vaccination strategies against human tumor Ags (13). One such tumor Ag is the unique, tumor-specific variable region Ig expressed by malignant B cells known as the idiotype (Id). Ig idiotypic sequences have been shown to contain epitopes capable of being recognized by Abs (14–17), CD4<sup>+</sup> T cells (16, 18–20), or CD8<sup>+</sup> T cells (21–23). Vaccination of B cell lymphoma patients with tumor-derived Id protein coupled to the highly immunogenic foreign carrier protein KLH (Id-KLH) along with a chemical adjuvant can result in anti-Id immune responses that correlate with prolonged time to disease progression and survival (19, 24). However, such immune responses are not elicited in all patients and are primarily humoral. In seeking to improve the frequency and potency of humoral and cellular anti-Id immune

responses in lymphoma patients, DCs were chosen because of their unique capacity to stimulate cellular immunity. Ig Id was the first human tumor Ag to be evaluated by pulsed DC vaccination (18). In a pilot clinical trial of Id-pulsed DC vaccination for B cell lymphoma, objective tumor regressions have been observed in 3 of 10 patients vaccinated with peripheral blood DCs pulsed with Id protein (18, 25). To further refine this approach, we have sought to improve the immunogenicity of Id-pulsed DC vaccines using modified Ags for DC pulsing in a well-characterized murine B cell lymphoma model. A chimeric form of Id linked to GM-CSF was chosen for DC pulsing, because vaccination with this protein had been previously shown to elicit protective anti-tumor immunity without the need for exogenous adjuvants (26). These effects are believed to rely in part on the stimulatory effects of GM-CSF on DCs (27, 28). Id chemically linked to KLH was also used, because of its known immunogenicity in a number of different Id vaccine formulations (19, 24, 29, 30). We have now shown that vaccination with DCs pulsed with native, unmodified Id protein fails to elicit a detectable anti-tumor immune response. In contrast, Id proteins modified by linkage to foreign constant regions, foreign constant regions plus GM-CSF, or KLH carrier protein were increasingly potent in their ability to generate anti-Id Abs when used for pulsed DC vaccination. Vaccination with DCs pulsed with Id coupled to KLH induced tumor-protective immunity superior to that obtained with a chemical adjuvant, and the protection was not dependent on effector T cells.

## Materials and Methods

### Mice and cell lines

Six- to 8-wk-old female C3H/HeN mice were obtained from Harlan Sprague-Dawley (San Diego, CA) and were housed at the Laboratory Animal Facility at Stanford University Medical Center (Stanford, CA). The carcinogen-induced murine B cell lymphoma 38C13 expressing a clonal IgM/ $\kappa$  on its surface has been previously described (16). 38C13 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 50  $\mu$ M 2-ME (cRPMI-10). All media and supplements were obtained from Life Technologies/BRL (Frederick, MD).

Division of Oncology, Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305

Received for publication October 28, 1999. Accepted for publication February 16, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> This work was supported by National Institutes of Health-U.S. Public Health Service Grant CA33399. J.M.T. was a Fellow of the Cancer Research Institute and a recipient of the American Society of Clinical Oncology Young Investigator Award. R.L. is an American Cancer Society Clinical Research Professor.

<sup>2</sup> Address correspondence and reprint requests to Dr. Ronald Levy, Department of Medicine, Division of Oncology, Stanford University Medical Center, Room M207, Stanford, CA 94305. E-mail address: levy@leland.stanford.edu

<sup>3</sup> Abbreviations used in this paper: DC, dendritic cell; Id, B cell lymphoma idiotype; KLH, keyhole limpet hemocyanin; HCR, human constant regions; Id-KLH, Id coupled to KLH.

### Generation and Ag pulsing of bone marrow-derived DC

Bone marrow-derived DCs were prepared as previously described (3) with minor modifications. Briefly, bone marrow was flushed from the femurs and tibias of mice and depleted of red cells by ammonium chloride lysis. Bone marrow cells were then depleted of T, B, and MHC class II<sup>+</sup> cells by treatment with rat mAbs followed by panning on plates coated with goat anti-rat Ig Abs (Southern Biotechnology Associates, Birmingham, AL). The mAbs used were GK1.5 (anti-CD4), 53-6.7 (anti-CD8) (TIB 207 and TIB 105, respectively, American Type Culture Collection, Manassas, VA), RA3-6B2 (anti-B220, PharMingen, San Diego, CA), and 10-3.6 (anti-I-A<sup>k</sup>, PharMingen). Cells were then plated in six-well culture plates at  $10^6$ /ml in cRPMI-10 without cytokines. After overnight culture, nonadherent cells were harvested and replated at  $2.5 \times 10^5$ /ml in cRPMI-10 supplemented with 10 mM HEPES, 1000 U/ml recombinant murine GM-CSF (provided by Immunex, Seattle, WA), and 1000 U/ml recombinant murine IL-4 (provided by Dr. Robert Coffman, DNAX, Palo Alto, CA). Medium was replenished (~50%) after 4–5 days as needed. After 6 days of culture, non-adherent and loosely adherent cells were harvested by gently pipetting and replated at  $10^6$  cells/ml in fresh medium with cytokines. Nonadherent DCs were harvested on the seventh day, replated at  $10^6$  cells/ml, and cultured overnight in the presence of medium alone or Ag at 100  $\mu$ g/ml. This concentration of Ag was chosen based on previous studies in several other murine tumor models (2, 6). Except where noted, pulsing medium was supplemented with 1000 U/ml GM-CSF. The following day, nonadherent and loosely adherent DCs were harvested, washed three times, and resuspended in HBSS.

### Production and modification of Id proteins

38C13 Id and an isotype-matched IgM/ $\kappa$  protein (4C5) were derived from tumor-myeloma cell hybridomas as previously described (16) and affinity purified using mannose binding protein columns (Pierce, Rockford, IL) to >95% purity as determined by SDS-PAGE. Proteins were coupled to KLH (Calbiochem, San Diego, CA) using glutaraldehyde as previously described (16). Chimeric Id protein, composed of 38C13 variable regions linked to human IgG1/ $\kappa$  constant regions (Id-HCR), and chimeric Id fusion protein containing murine GM-CSF at its C-terminus (Id-HCR-GM) were produced as previously described (26).

### Vaccinations

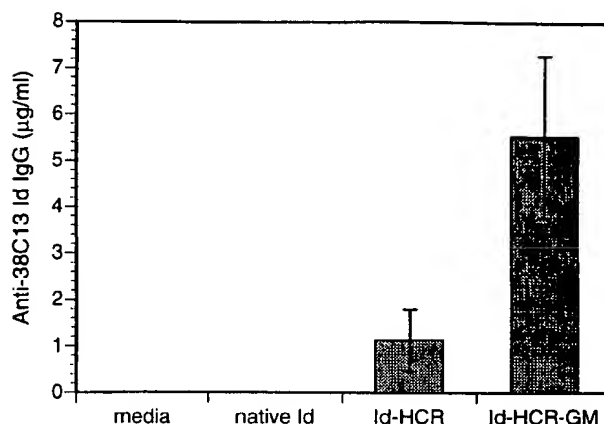
Washed, Ag-pulsed DCs were injected i.v. by tail vein in a volume of 0.1 ml of HBSS. In different experiments, the number of injected DCs varied between  $2.5 \times 10^5$ /mouse. DC vaccines were compared with Id-HCR-GM fusion protein (50  $\mu$ g) administered i.p. twice biweekly in PBS without additional adjuvants and to Id-KLH conjugate (50  $\mu$ g) given s.c. twice biweekly in PBS or together with QS21 adjuvant (31) (10  $\mu$ g) three times biweekly (QS21 was provided by Aquila Biopharmaceuticals, Framingham, MA).

### Humoral immune response assessments

Twelve to 14 days following the last immunization, blood was collected by tail vein. Serum anti-Id Abs were quantitated by ELISA as previously described (16). Briefly, 96-well Maxisorb plates (Nunc, Naperville, IL) were coated with 38C13 IgM and incubated with serially diluted immune sera. Bound Abs were detected with HRP-conjugated goat anti-mouse IgG (Caltag, Burlingame, CA) using the 2,2'-azino-bis-(3-ethyl)-benzthiazoline sulfonic acid substrate and absorbance determination at 405 nm with a Vmax microplate reader (Molecular Devices, Menlo Park, CA). A mixture of affinity-purified anti-38C13 Id mAbs containing IgG1, IgG2a, and IgG2b isotypes in a 2:1:1 ratio was used to generate a standard curve for total anti-Id IgG. Isotype-specific detectors were used for the quantitation of anti-Id Abs of the IgG1 and IgG2a subclasses (Southern Biotechnology Associates) using relevant isotype standards. Purified 4C5 (IgM/ $\kappa$ ) and BCL1 (IgM/ $\lambda$ ) Igs were used to check the specificity of the anti-Id response.

### Tumor challenge

38C13 tumor cells were thawed from a common dedicated frozen stock 2 days before tumor challenge and were split on the day before use. On the day of tumor challenge, cells were washed three times in serum-free RPMI and diluted to the appropriate concentration in HBSS. Groups of mice received either 1000 cells s.c. above the base of the tail or 200 cells i.p. in a volume of 0.2 ml. Thereafter, mice were followed daily for survival. Survival analysis was performed using GraphPad Prism software (San Diego, CA), and *p* values were calculated using the log-rank statistical test.



**FIGURE 1.** Humoral anti-idiotypic response after vaccination with Ag-pulsed DCs. Groups of mice were given two biweekly vaccinations of DCs pulsed with medium alone, native Id protein, chimeric Id protein (Id-HCR), or chimeric Id-GM-CSF fusion protein (Id-HCR-GM). Two weeks later sera were collected and analyzed for anti-38C13 Id IgG Abs by ELISA. Bars represent mean determinations in three animals, with error bars showing  $\pm$ SD. Results are representative of three individual experiments.

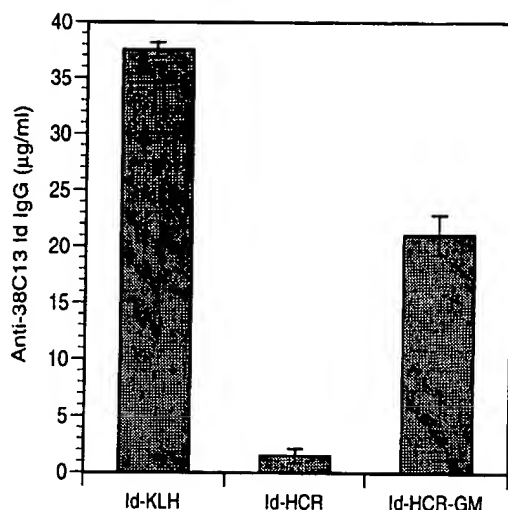
### In vivo depletion of T lymphocyte subsets

Groups of 10 mice were vaccinated three times biweekly with Id-KLH-pulsed DCs and challenged with tumor 2 wk later on day 0. On days -6, -5, -4, and 0 and weekly thereafter for 4 wk mice were injected i.p. with 200  $\mu$ g of T cell-depleting or control mAbs (from ascites). Abs used were the CD4<sup>+</sup> T cell-depleting mAb GK1.5 (rat IgG2b), control rat IgG2a mAb H22-15-5, CD8<sup>+</sup> T cell-depleting mAb HB129 (mouse IgG2a), or control mouse IgG2a mAb 17F12. On day -1 with respect to tumor challenge, peripheral blood was collected from two representative mice from each group, and lymphocytes were analyzed by flow cytometry for depletion of the appropriate T cell subset. Staining Abs CT-CD4 (anti-CD4, Caltag) and 53-5.8 (anti-CD8 $\beta$ 2, PharMingen) were not cross-blocked by the depleting Abs and demonstrated >98% depletion of the target T cell population.

## Results

### Immunogenicity of DCs pulsed with native, chimeric, or Id-GM-CSF fusion proteins

To identify an Ag pulsing strategy that would lead to greater immunogenicity of tumor Ag-pulsed DC vaccines, we examined the use of modified tumor Ag (Id) proteins for DC pulsing. To evaluate this approach we chose bone marrow-derived DCs grown in GM-CSF and IL-4. Such DCs have been shown to take up and process soluble protein Ags (6, 8, 27). For the Ag, we first examined recombinant forms of the 38C13 Id containing xenogeneic constant region and cytokine sequences, since in previous studies these proteins had displayed increased immunogenicity over that of native Id (26). We compared the immunogenicity of DCs pulsed with native Id, a chimeric Id protein containing xenogeneic (human) constant region sequences (Id-HCR), or a chimeric Id protein also having murine GM-CSF linked to its C terminus (Id-HCR-GM) (26). Mice received two biweekly i.v. injections of Ag- or medium-pulsed DCs. Following vaccination we monitored the humoral anti-Id response by ELISA (16). The measurement of anti-Id Abs is highly relevant in this tumor model, as previous studies have demonstrated that the induction of humoral anti-Id immunity was sufficient to provide protection from tumor challenge (16, 29, 32). After two immunizations, there was no detectable humoral response in mice vaccinated with DCs pulsed with native Id protein (Fig. 1). Low levels of anti-38C13 Id IgG were detected in mice given DCs pulsed with the chimeric Id, yet substantially higher levels were elicited by DCs pulsed with the chimeric GM-CSF fusion protein. Administration of DCs pulsed with an irrelevant

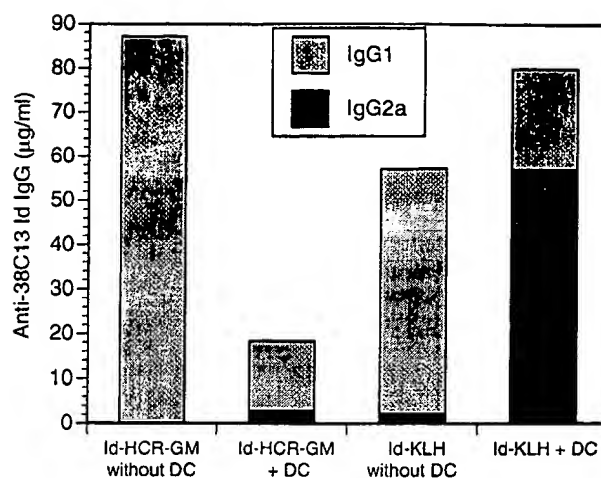


**FIGURE 2.** Humoral anti-idiotype response after vaccination with DCs pulsed with KLH-coupled Id, chimeric Id, or chimeric Id-GM-CSF fusion proteins. Groups of mice were given three biweekly vaccinations of DCs pulsed with Id protein chemically coupled to KLH (Id-KLH), chimeric Id protein (Id-HCR), or chimeric Id-GM-CSF fusion protein (Id-HCR-GM). Two weeks later sera were collected and analyzed for anti-38C13 Id IgG Abs by ELISA. Bars represent mean determinations in three animals, with error bars showing  $\pm$ SD. Results are representative of three individual experiments.

tumor Id (BCL-1) chimeric GM-CSF fusion protein failed to elicit Abs specific for the 38C13 Id (data not shown). GM-CSF is known to enhance the uptake and processing of Ag by DCs (6, 28). Therefore, we next asked whether excess soluble GM-CSF present during the pulsing period could substitute for the cytokine moiety linked directly to Id in the Id-HCR-GM fusion protein. Soluble GM-CSF (200 ng/ml = 20-fold excess of that which supports DC growth and differentiation *in vitro*) added during overnight Ag pulsing could not enhance the humoral anti-Id response to DCs pulsed with the chimeric Id to the level attained when using Ag directly fused to the cytokine ( $0.62 \pm 0.39$  vs  $2.7 \pm 1.59$   $\mu$ g/ml), nor could it render native Id-pulsed DCs immunogenic.

#### *Immunogenicity of DC pulsed with Id coupled to the KLH carrier protein*

Linkage of xenogeneic carrier proteins such as KLH to weak, self-derived tumor Ags can enhance their immune recognition (16). We next asked whether the chemical coupling of KLH to Id could enhance its immunogenicity when used for DC pulsing. Mice received three biweekly i.v. injections of DCs pulsed with medium alone, Id-HCR, Id-HCR-GM, or Id-KLH. Immunization of mice with DCs pulsed with Id-KLH generated a surprisingly high anti-Id response, surpassing even that of DCs pulsed with Id-HCR-GM (Fig. 2). Vaccination with DCs pulsed with an unrelated control IgM/ $\kappa$  (4C5) coupled to KLH failed to elicit any anti-38C13-specific Abs (data not shown). The enhanced immunogenicity of Id-KLH-pulsed DCs was not simply due to aggregation of the Id, as the use of Id conjugated to itself for DC pulsing yielded no anti-Id response. Likewise, concurrent administration of an equivalent number of DCs pulsed only with KLH along with DCs pulsed with native Id failed to elicit a response, demonstrating the inability of carrier-pulsed DCs to supply sufficient helper activity to coadministered Id-pulsed DCs for stimulation of an anti-Id humoral response (data not shown).



**FIGURE 3.** Isotype profiles of anti-Id Abs elicited by various vaccination methods. Groups of three mice were vaccinated with soluble Id-GM-CSF fusion protein (Id-HCR-GM), Id-KLH, or DCs pulsed with Id-HCR-GM or Id-KLH as described in *Materials and Methods*. Two weeks later sera were collected and pooled, and 38C13 Id-specific Abs were quantitated by ELISA using isotype-specific detectors. Results are representative of two experiments.

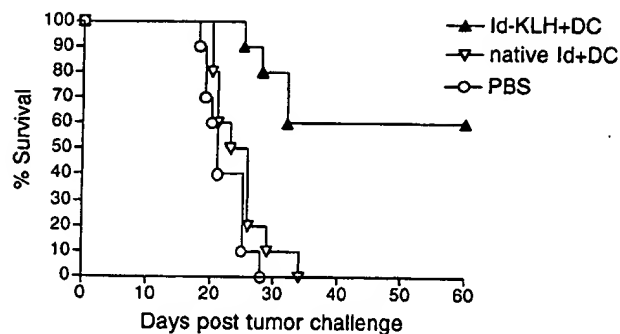
#### *Vaccination with DCs pulsed with modified Id proteins induces Ab isotype profiles consistent with a Th1 response*

To further characterize the anti-Id immune response to vaccination with DCs pulsed with modified Id proteins, we examined the isotype profiles of IgG anti-Id Abs following vaccination (Fig. 3). Vaccination of mice with chimeric Id-HCR-GM fusion protein without DCs induced high levels of anti-Id Abs exclusively of the IgG1 isotype. In contrast, vaccination with DCs pulsed with Id-HCR-GM induced lower levels of Abs, but with substantial proportions of the IgG2a isotype. Similarly, vaccination with Id-KLH alone led to a primarily IgG1 anti-Id response, while vaccination with Id-KLH-pulsed DCs led to high titers of anti-Id Abs with a striking predominance of the IgG2a isotype characteristic of a Th1-type response (33). These data argue against carryover of soluble protein Ag by the DCs as the sole mechanism for induction of the humoral anti-Id response, because the responses to Ag-pulsed DCs differ qualitatively from those elicited by injection of the same proteins without DCs.

#### *Tumor protection following vaccination with DCs pulsed with Id-KLH*

Given the superior humoral anti-Id immune response generated by vaccination with Id-KLH-pulsed DCs, we tested vaccinated mice for resistance to challenge with 38C13 lymphoma cells (Fig. 4). Mice vaccinated with DCs pulsed with native Id protein and challenged s.c. with 1000 38C13 tumor cells all died within 5 wk and were not protected compared with those receiving PBS. However, a majority of mice vaccinated with Id-KLH-pulsed DCs survived the tumor challenge ( $p < 0.0001$  and  $p < 0.0005$  for Id-KLH-pulsed DCs vs PBS and native Id-pulsed DCs, respectively). Comparable results were obtained in two additional experiments. No significant protection from tumor challenge was observed following vaccination with DCs pulsed with Id conjugated to itself or with an irrelevant IgM/ $\kappa$  (4C5) coupled to KLH (data not shown).

Next, we wished to compare the efficacy of vaccination with Id-KLH-pulsed DCs to that of a standard approach to Id vaccination, that is, vaccination with Id-KLH protein administered s.c. along with a chemical adjuvant (QS21). Mice given three biweekly



**FIGURE 4.** Survival following vaccination with DCs pulsed with native vs KLH-conjugated Id protein. Groups of 10 mice were given three bi-weekly injections of PBS or DCs pulsed with native Id protein or Id-KLH. Two weeks later mice were injected s.c. with 1000 38C13 tumor cells and followed for survival. Results are representative of three individual experiments.

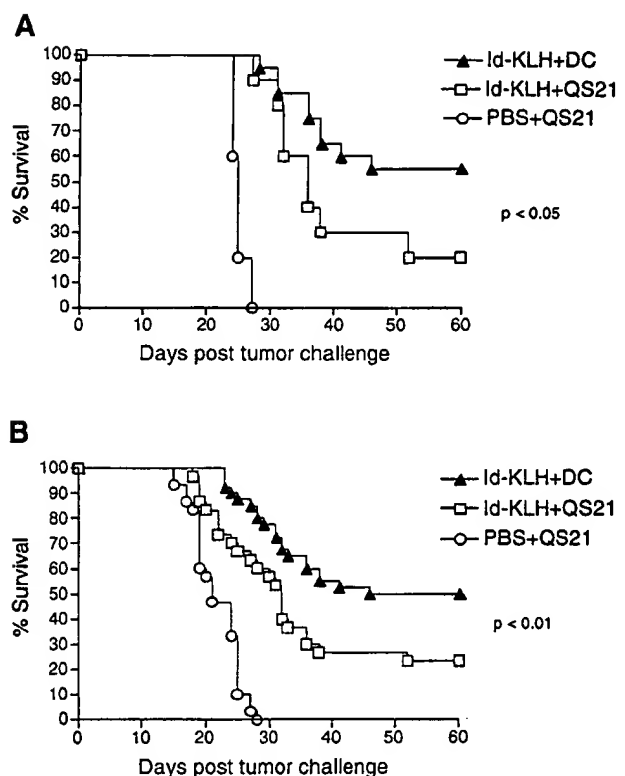
injections of either Id-KLH plus QS21 or Id-KLH-pulsed DCs developed high serum titers of anti-Id Abs (mean, 210 and 186  $\mu\text{g/ml}$ , respectively, in a representative experiment), with a dominance of the IgG2a isotype. Animals vaccinated with Id-KLH plus QS21 displayed prolonged survival compared with animals given PBS plus QS21, yet only a minority of the animals survived long term (Fig. 5A). However, vaccination with Id-KLH-pulsed DCs provided superior protection over Id-KLH plus QS21, with the majority of animals surviving  $>60$  days ( $p = 0.047$ ). Analogous results were obtained in two additional experiments. Fig. 5B shows the pooled survival results of the three independent experiments and demonstrates the consistent advantage of Id-KLH-pulsed DCs over Id-KLH plus chemical adjuvant in providing tumor protection ( $p = 0.0082$ ).

#### Role of effector T cells in tumor protection

We next sought to determine the contribution of effector T cells to tumor protection in mice vaccinated with Id-KLH-pulsed DCs. Groups of mice given three biweekly vaccinations with Id-KLH-pulsed DCs were treated with mAbs to deplete effector CD4<sup>+</sup> or CD8<sup>+</sup> T cells before and following tumor challenge as described in *Materials and Methods* (Fig. 6). Depletion of each T cell subset from the peripheral blood of vaccinated mice was confirmed by flow cytometry using non-cross-blocking anti-CD4 and anti-CD8 mAbs before tumor challenge (data not shown). Depletions were maintained throughout the experiment by continued weekly administration of the depleting mAbs. The isotype-matched control mAbs used had no significant effect on the relevant T cell population. Mice depleted of CD4<sup>+</sup> or CD8<sup>+</sup> T cells following vaccination with Id-KLH-pulsed DCs had no statistically significant alterations in survival compared with mice receiving isotype-matched control mAbs ( $p = 0.325$  and  $p = 0.136$ , respectively) or compared with those receiving no mAbs ( $p = 0.802$  and  $p = 0.107$ , respectively).

#### Discussion

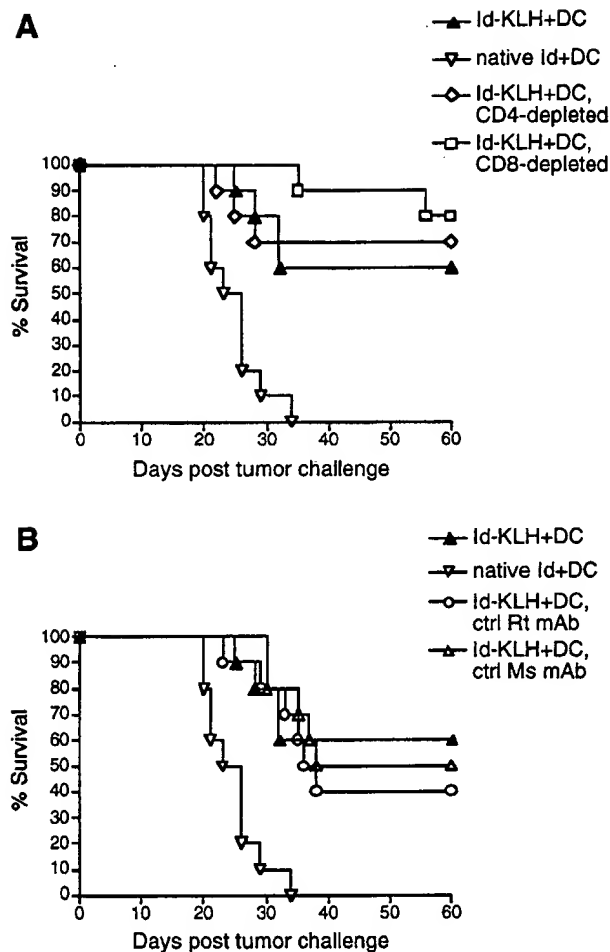
There is currently great interest in exploiting the powerful Ag-presenting functions of DCs to elicit therapeutic anti-tumor immunity (1). Studies in murine tumor model systems have provided the basis for numerous clinical trials of vaccination using DCs pulsed with tumor Ag-derived peptides, proteins, RNA, or tumor cell preparations (13). Clinical trials in B cell lymphoma using native Id protein-pulsed DCs have yielded promising initial results, with objective tumor regressions being observed in 3 of 10 cases, in-



**FIGURE 5.** Survival following vaccination with DCs pulsed with Id-KLH vs vaccination with Id-KLH plus chemical adjuvant (QS21). *A*, Groups of mice were given three biweekly injections of Id-KLH-pulsed DCs ( $n = 20$ ), Id-KLH plus QS21 adjuvant ( $n = 10$ ), or PBS plus QS21 ( $n = 10$ ). Two weeks later mice were injected s.c. with 1000 38C13 tumor cells and followed for survival. *B*, Pooled survival of mice vaccinated with Id-KLH-pulsed DCs ( $n = 40$ ) or Id-KLH plus QS21 adjuvant ( $n = 30$ ) in three separate experiments is shown. Tumor challenge cells were administered i.p. (200 cells) in one of the three experiments ( $n = 20$  mice) and s.c. (1000 cells) in two experiments ( $n = 50$  mice).

cluding two durable complete responses (18, 24) (J. Timmerman and R. Levy, manuscript in preparation). However, preclinical studies of protein tumor Ag-pulsed DC vaccination have been limited (2, 6, 8), and an optimal method for Ag pulsing has not been defined. We have thus sought to refine tumor Ag-pulsed DC vaccination using a well-characterized murine tumor expressing a weak, self-derived tumor Ag (38C13 Id) (16). In this report, we have shown that 38C13 Id-pulsed DCs can generate a humoral anti-Id response only when the Ag is modified to constitute a hapten-carrier system. Id proteins modified by inclusion of foreign constant regions, foreign constant regions plus GM-CSF, or linkage to KLH carrier protein were increasingly potent in their ability to generate anti-Id Abs following pulsed DC vaccination. DCs functioned as an efficient adjuvant for induction of the anti-Id humoral response and elicited predominantly IgG2a Abs characteristic of a Th1 response when Id-KLH was used for DC pulsing. Id-KLH-pulsed DC vaccination was significantly more effective in inducing protective immunity than vaccination with the same Ag together with a chemical adjuvant. Surprisingly, despite the deliberate attempt to induce a cellular anti-Id response through the use of DCs, tumor protection afforded by this particular vaccine was not dependent on effector T cells.

We chose bone marrow-derived DCs cultured in GM-CSF and IL-4 for this study, as these cells resemble the monocyte-derived



**FIGURE 6.** Effects of in vivo depletion of T cell subsets on survival following vaccination with Id-KLH-pulsed DCs. Groups of 10 mice were vaccinated three times biweekly with Id-KLH-pulsed DCs, then challenged 2 wk later (day 0) with 1000 38C13 tumor cells s.c. and followed for survival. On days -6, -5, -4, and 0 weekly thereafter mice were treated with CD4<sup>+</sup> T cell-depleting mAb GK1.5 or CD8<sup>+</sup> T cell-depleting mAb HB129 (*A*) or with isotype control rat (ctrl Rt) or mouse (ctrl Ms) mAbs (*B*). Survival of animals vaccinated with native Id-pulsed DCs is shown as a negative control for comparison.

DCs most widely used in clinical trials of DC vaccination for cancer (13, 28). In concordance with our clinical studies (18, 25), vaccination of mice with native Id-pulsed DCs failed to elicit a humoral anti-Id response (Fig. 1). However, in our mouse model, the addition of xenogeneic constant region sequences to the Id endowed it with the capacity to stimulate an anti-Id humoral response when used for DC pulsing (Figs. 1 and 2). This was presumably due to the introduction of foreign helper epitopes, thus creating a hapten-carrier system (34). The addition of GM-CSF sequence to this chimeric Id protein further augmented the immunogenicity of the pulsed DC vaccine, as judged by the humoral response assay (Figs. 1 and 2). The mechanism of the GM-CSF moiety's effect is probably due to stimulation of the Ag uptake or Ag presentation functions of the DCs (28) rather than to the contribution of additional helper epitopes, because a chimeric Id fusion protein containing human GM-CSF (which is not biologically active in the mouse) does not show enhanced immunogenicity over that of chimeric Id alone (data not shown). The inability of soluble, unlinked GM-CSF to boost the immunogenicity of the chimeric Id

to that of the Id-HCR-GM fusion protein may also suggest targeting of the GM-CSF fusion protein to the DCs by binding to the GM-CSF receptor.

We expected that anti-Id T cell responses would be detectable following Id-pulsed DC vaccination. Bone marrow-derived DCs cultured in GM-CSF and IL-4 have been previously shown to induce protective anti-tumor immunity mediated by CD8<sup>+</sup> T cells when pulsed with the model protein tumor Ags  $\beta$ -galactosidase and OVA (6, 8). Indeed, we confirmed that OVA-pulsed DCs could readily induce cytolytic activity toward OVA-expressing E.G7 cells, yet under analogous conditions no Id-specific CTL activity could be generated against 38C13 lymphoma cells (data not shown). In addition, based upon the in vivo T cell depletion data, CD4<sup>+</sup> or CD8<sup>+</sup> T cells appear to play no significant role as effectors of tumor protection in mice vaccinated with Id-KLH-pulsed DCs (Fig. 6).

Our findings underscore the important role of DCs in the generation of humoral immune responses. DCs have long been known to be critical for the development of Ab responses to T-dependent Ags by priming Ag-specific Th cells to secrete soluble B cell stimulatory factors (35, 36) and up-regulate CD40 ligand (37). However, a more complex role for DCs in the humoral response was suggested by observations of their clustering with B cells both in vitro (36) and in vivo (38). In some of the earliest studies of Ag-pulsed DC vaccination, injection of mice with splenic DCs pulsed with viral particles or soluble protein Ag was found to stimulate a specific Ab response without the need for additional adjuvants (2, 39, 40). The Abs induced included significant proportions of the IgG2a isotype (2, 40), indicative of a Th1 response (33). These findings suggested the transfer of native, unprocessed Ag to B cells, although the nature of such Ag handling by DCs was not defined. Recently, Wykes et al. demonstrated the transfer of native protein Ag from DCs to B cells to generate a class-switched humoral response (41). Splenic DCs pulsed with DNP-KLH were shown to take up and retain unprocessed Ag in an intracellular compartment for at least 48 h. Induction of DNP-specific IgG required DC to be intact and in direct contact with the responding B cells. Dubois and colleagues (42) have provided further evidence for the direct signaling of DCs to B cells with the demonstration that DCs could stimulate both proliferation and Ab secretion by CD40-activated B cells. These effects are dependent on direct DC-B cell interactions as well as soluble factors, including IL-12 (43). The recent identification of the novel B cell-specific growth and differentiation factor, BAFF, as a product of DCs also confirms the direct influence of DCs over the humoral immune response (44).

A role of DCs as vehicles for the delivery of native, unprocessed Ags to B cells explains the efficiency of DCs in augmenting the humoral anti-Id responses observed in our study. As the Id-KLH pulsed DCs were washed extensively before injection, the amount of Ag delivered would be expected to be small relative to that of a protein-plus-adjuvant vaccine, yet the pulsed DCs elicited similar Ab titers (Fig. 3). Prior reports of humoral response induction by Ag-pulsed DCs have made use of splenic DCs (2, 35, 36, 39–41). The present study is the first description of bone marrow-derived DCs pulsed with tumor Ag eliciting a protective humoral immune response. Thus, it appears that in vitro-generated DCs can also serve as efficient vehicles for the delivery of Ag to prime a humoral immune response.

The mechanisms of protective tumor immunity following anti-Id vaccination have been proposed to include both Abs (16, 17, 29, 32) and T cell effectors (16, 30, 45–48) in various murine lymphoma (16, 17, 29, 30, 32, 47) and myeloma (45, 46, 48) models.

Early investigations into the protective mechanisms of 38C13 tumor immunity following vaccination with Id-KLH plus chemical adjuvants suggested a role for CD4<sup>+</sup> and CD8<sup>+</sup> T cells based on in vivo depletion experiments (29). However, immune serum transfer studies clearly demonstrated that Abs could be sufficient for tumor protection (29). More recent studies in the 38C13 lymphoma model using Id-encoding plasmid DNA vaccination have failed to demonstrate any role for effector T cells in tumor protection despite the general propensity of DNA vaccination to elicit T cell immunity and, in particular, CTL (32). In addition, direct cytotoxicity of Id-immune CTL toward 38C13 tumor cells has never been demonstrated (29, 30, 32, 47). The present study provides no exception, as no dependence on T cell effectors was observed (Fig. 6). Nonetheless, it remains possible that under certain vaccination conditions, T cell effectors may contribute to immunity against 38C13 Id (30, 47). In humans, the ability to elicit anti-Id T cell immunity is likely to depend on an individual tumor's Id sequences and the host haplotype. Some individual Ids may contain sequences capable of eliciting anti-Id CD4<sup>+</sup> and/or CD8<sup>+</sup> T cells, while others may not. This variation may account for the finding of anti-Id cellular proliferative responses in only 50% of lymphoma patients vaccinated with DCs pulsed with tumor-derived Id (25).

Because T cells were not found to mediate tumor protection following Id-KLH-pulsed DC vaccination in this model, anti-Id Abs are likely to be necessary effectors. Vaccination using both DCs and the QS21 adjuvant elicited high titers of anti-Id Abs. However, tumor protection was superior with the use of DCs (Fig. 5). Delivery of Ag by DCs resulted in a higher proportion of Abs of the IgG2a isotype, possibly due to DC-derived IL-12 favoring the development of a Th1 response and its associated isotype profile (43, 49, 50). Higher titers of anti-Id Abs of the IgG2a subclass may contribute to the greater protection seen with pulsed DC vaccination. In studies of passive anti-Id mAb therapy for 38C13 lymphoma, anti-Id Abs of the IgG2a subclass were up to 100-fold more potent in conferring tumor protection than their class-switched IgG1 counterparts (51). However, we found no correlation between the serum anti-Id levels of individual mice and their degree of tumor protection in the current study (data not shown). This suggests that another rate-limiting effector function (such as Ab-dependent cellular cytotoxicity) may have been more efficiently induced by DC vaccination.

Our findings differ from those of Flamand et al. (2), who describe the induction of anti-Id Abs and protective immunity against the BCL-1 murine lymphoma following vaccination with native BCL-1 Id-pulsed splenic DCs. In contrast, we observed that conjugation of 38C13 Id protein to KLH was required to elicit both anti-Id Abs and tumor protection. However, there are several important differences between these two studies. First, Flamand et al. used splenic DCs, whereas in the current study bone marrow-derived DCs were used. Secondly, the BCL-1 and 38C13 variable region sequences share little homology and may thus differ significantly in their content of helper epitopes capable of priming CD4<sup>+</sup> T cells required for efficient induction of the humoral anti-Id response. Furthermore, in the current study we demonstrated that DCs can function as a superior adjuvant for the induction of protective immunity against the 38C13 tumor compared with the chemical adjuvant QS21 (Fig. 5), while Flamand et al. did not demonstrate an advantage of BCL-1-pulsed DCs over Id protein administered s.c. in CFA.

Despite the expectation that DCs would favor the generation of anti-Id T cell immunity, we have found the tumor-protective effects of Id-KLH-pulsed DC vaccination to be dependent largely, if not exclusively, on the humoral anti-Id response in the 38C13 tumor model. In the case of Id vaccination for B cell lymphomas,

induction of a humoral response is desirable given the proven anti-tumor effects of anti-Id Abs (14, 17, 29, 32, 52). Humoral anti-tumor responses may also be advantageous against other cancers that are susceptible to Ab-mediated control. As such, our findings provide rationale for the use of KLH-conjugated Id for DC pulsing in lymphoma clinical trials and suggest a strategy with possible implications for DC vaccination against other human cancers.

## Acknowledgments

We thank Carmen Wong, Shoshana Levy, and Stacie Lambert for their critical reading of the manuscript. We are also grateful to Elaine Thomas of Immunex and Robert Coffman of DNAX for generously providing cytokine reagents.

## References

- Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392:245.
- Flamand, V., T. Sornasse, K. Thielemans, C. Demanet, M. Bakkus, H. Bazin, F. Thielemans, O. Leo, J. Urbain, and M. Moser. 1994. Murine dendritic cells pulsed in vitro with tumor antigen induce tumor resistance in vivo. *Eur. J. Immunol.* 24:605.
- Mayordomo, J. I., T. Zorina, W. J. Storkus, L. Zitvogel, C. Celluzzi, L. D. Falo, C. J. Melief, S. T. Ildstad, W. M. Kast, A. B. DeLeo, et al. 1995. Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumor immunity. *Nat. Med.* 1:1297.
- Zitvogel, L., J. I. Mayordomo, T. Tjandrawan, A. B. DeLeo, M. R. Clarke, M. T. Lotze, and W. J. Storkus. 1996. Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. *J. Exp. Med.* 183:87.
- Celluzzi, C. M., J. I. Mayordomo, W. J. Storkus, M. T. Lotze, and L. D. Falo, Jr. 1996. Peptide-pulsed dendritic cells induce antigen-specific CTL-mediated protective tumor immunity. *J. Exp. Med.* 183:283.
- Paglia, P., C. Chiodoni, M. Rodolfo, and M. P. Colombo. 1996. Murine dendritic cells loaded in vitro with soluble protein prime cytotoxic T lymphocytes against tumor antigen in vivo. *J. Exp. Med.* 183:317.
- Porgador, A., and E. Gilboa. 1995. Bone marrow-generated dendritic cells pulsed with a class I-restricted peptide are potent inducers of cytotoxic T lymphocytes. *J. Exp. Med.* 182:255.
- Porgador, A., D. Snyder, and E. Gilboa. 1996. Induction of antitumor immunity using bone marrow-generated dendritic cells. *J. Immunol.* 156:2918.
- Nair, S. K., D. Boczkowski, D. Snyder, and E. Gilboa. 1997. Antigen-presenting cells pulsed with unfractionated tumor-derived peptides are potent tumor vaccines. *Eur. J. Immunol.* 27:589.
- Song, W., H. L. Kong, H. Carpenter, H. Torii, R. Granstein, S. Rafii, M. A. Moore, and R. G. Crystal. 1997. Dendritic cells genetically modified with an adenovirus vector encoding the cDNA for a model antigen induce protective and therapeutic antitumor immunity. *J. Exp. Med.* 186:1247.
- Specht, J. M., G. Wang, M. T. Do, J. S. Lam, R. E. Royal, M. E. Reeves, S. A. Rosenberg, and P. Hwu. 1997. Dendritic cells retrovirally transduced with a model antigen gene are therapeutically effective against established pulmonary metastases. *J. Exp. Med.* 186:1213.
- Boczkowski, D., S. K. Nair, D. Snyder, and E. Gilboa. 1996. Dendritic cells pulsed with RNA are potent antigen-presenting cells in vitro and in vivo. *J. Exp. Med.* 184:465.
- Timmerman, J. M., and R. Levy. 1999. Dendritic cell vaccines for cancer immunotherapy. *Annu. Rev. Med.* 50:507.
- Miller, R. A., D. G. Maloney, R. Wamke, and R. Levy. 1982. Treatment of B-cell lymphoma with monoclonal anti-idiotypic antibody. *N. Engl. J. Med.* 306:517.
- Miller, R. A., S. Hart, M. Samoszuk, C. Coulter, S. Brown, D. Czerwinski, J. Kelkenberg, I. Royston, and R. Levy. 1989. Shared idiotypes expressed by human B-cell lymphomas. *N. Engl. J. Med.* 321:851.
- Campbell, M. J., W. Carroll, S. Kon, K. Thielemans, J. B. Rothbard, S. Levy, and R. Levy. 1987. Idiotypic vaccination against murine B cell lymphoma: humoral and cellular responses elicited by tumor-derived immunoglobulin M and its molecular subunits. *J. Immunol.* 139:2825.
- George, A. J., A. L. Tutt, and F. K. Stevenson. 1987. Anti-idiotypic mechanisms involved in suppression of a mouse B cell lymphoma, BCL1. *J. Immunol.* 138:628.
- Hsu, F. J., C. Benike, F. Fagnoni, T. M. Liles, D. Czerwinski, B. Taidi, E. G. Engleman, and R. Levy. 1996. Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat. Med.* 2:52.
- Kwak, L. W., M. J. Campbell, D. K. Czerwinski, S. Hart, R. A. Miller, and R. Levy. 1992. Induction of immune responses in patients with B-cell lymphoma against the surface-immunoglobulin idiotype expressed by their tumors. *N. Engl. J. Med.* 327:1209.
- Lauritszen, G. F., S. Weiss, Z. Dembic, and B. Bogen. 1994. Naive idiotype-specific CD4<sup>+</sup> T cells and immunosurveillance of B-cell tumors. *Proc. Natl. Acad. Sci. USA* 91:5700.
- Chakrabarti, D., and S. K. Ghosh. 1992. Induction of syngeneic cytotoxic T lymphocytes against a B cell tumor. II. Characterization of anti-idiotypic CTL lines and clones. *Cell. Immunol.* 144:443.



22. Cao, W., B. A. Myers-Powell, and T. J. Braciale. 1994. Recognition of an immunoglobulin VH epitope by influenza virus-specific class I major histocompatibility complex-restricted cytolytic T lymphocytes. *J. Exp. Med.* 179:195.
23. Abe, A., N. Emi, H. Taji, M. Kasai, A. Kohno, and H. Saito. 1996. Induction of humoral and cellular anti-idiotypic immunity by intradermal injection of naked DNA encoding a human variable region gene sequence of an immunoglobulin heavy chain in a B cell malignancy. *Gene Ther.* 3:988.
24. Hsu, F. J., C. B. Caspar, D. Czerwinski, L. W. Kwak, T. M. Liles, A. Syrengelas, B. Taidi-Laskowski, and R. Levy. 1997. Tumor-specific idiotype vaccines in the treatment of patients with B-cell lymphoma: long-term results of a clinical trial. *Blood* 89:3129.
25. Timmerman, J. M., T. A. Davis, F. Hsu, C. Benike, T. M. Liles, D. Czerwinski, B. Taidi, A. V. Beckhove, E. Engleman, and R. Levy. 1998. Clinical trials of dendritic cell vaccination for B cell lymphoma. *J. Leukocyte Biol.* 2(Suppl.):15.
26. Tao, M. H., and R. Levy. 1993. Idiotype/granulocyte-macrophage colony-stimulating factor fusion protein as a vaccine for B-cell lymphoma. *Nature* 362:755.
27. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R. M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176:1693.
28. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor  $\alpha$ . *J. Exp. Med.* 179:1109.
29. Campbell, M. J., L. Esserman, N. E. Byars, A. C. Allison, and R. Levy. 1990. Idiotype vaccination against murine B cell lymphoma: humoral and cellular requirements for the full expression of antitumor immunity. *J. Immunol.* 145:1029.
30. Kwak, L. W., H. A. Young, R. W. Pennington, and S. D. Weeks. 1996. Vaccination with syngeneic, lymphoma-derived immunoglobulin idiotype combined with granulocyte/macrophage colony-stimulating factor primes mice for a protective T-cell response. *Proc. Natl. Acad. Sci. USA* 93:10972.
31. Newman, M. J., J. Y. Wu, B. H. Gardner, K. J. Munroe, D. Leombruno, J. Recchia, C. R. Kensil, and R. T. Coughlin. 1992. Saponin adjuvant induction of ovalbumin-specific CD8<sup>+</sup> cytotoxic T lymphocyte responses. *J. Immunol.* 148:2357.
32. Syrengelas, A. D., and R. Levy. 1999. DNA vaccination against the idiotype of a murine B cell lymphoma: mechanism of tumor protection. *J. Immunol.* 162:4790.
33. Snapper, C. M., and J. J. Mond. 1993. Towards a comprehensive view of immunoglobulin class switching. *Immunol. Today* 14:15.
34. Berzofsky, J. A., and I. J. Berkower. 1993. Immunogenicity and antigen structure. In *Fundamental Immunology*. W. E. Paul, ed. Raven Press, New York, p. 235.
35. Inaba, K., A. Granelli-Piperno, and R. M. Steinman. 1983. Dendritic cells induce T lymphocytes to release B cell-stimulating factors by an interleukin 2-dependent mechanism. *J. Exp. Med.* 158:2040.
36. Inaba, K., M. D. Witmer, and R. M. Steinman. 1984. Clustering of dendritic cells, helper T lymphocytes, and histocompatible B cells during primary antibody responses in vitro. *J. Exp. Med.* 160:858.
37. Noelle, R. J., J. A. Ledbetter, and A. Aruffo. 1992. CD40 and its ligand, an essential ligand-receptor pair for thymus-dependent B-cell activation. *Immunol. Today* 13:431.
38. Kushnir, N., L. Liu, and G. G. MacPherson. 1998. Dendritic cells and resting B cells form clusters in vitro and in vivo: T cell independence, partial LFA-1 dependence, and regulation by cross-linking surface molecules. *J. Immunol.* 160:1774.
39. Francotte, M., and J. Urbain. 1985. Enhancement of antibody response by mouse dendritic cells pulsed with tobacco mosaic virus or with rabbit antiidiotypic antibodies raised against a private rabbit idio type. *Proc. Natl. Acad. Sci. USA* 82:8149.
40. Sornasse, T., V. Flamand, G. De Becker, H. Bazin, F. Tielemans, K. Thielemans, J. Urbain, O. Leo, and M. Moser. 1992. Antigen-pulsed dendritic cells can efficiently induce an antibody response in vivo. *J. Exp. Med.* 175:15.
41. Wykes, M., A. Pombo, C. Jenkins, and G. G. MacPherson. 1998. Dendritic cells interact directly with naive B lymphocytes to transfer antigen and initiate class switching in a primary T-dependent response. *J. Immunol.* 161:1313.
42. Dubois, B., B. Vanbervliet, J. Fayette, C. Massacrier, C. Van Kooten, F. Briere, J. Banchemau, and C. Caux. 1997. Dendritic cells enhance growth and differentiation of CD40-activated B lymphocytes. *J. Exp. Med.* 185:941.
43. Dubois, B., C. Massacrier, B. Vanbervliet, J. Fayette, F. Briere, J. Banchemau, and C. Caux. 1998. Critical role of IL-12 in dendritic cell-induced differentiation of naive B lymphocytes. *J. Immunol.* 161:2223.
44. Schneider, P., F. MacKay, V. Steiner, K. Hofmann, J. L. Bodmer, N. Holler, C. Ambrose, P. Lawton, S. Bixler, H. Acha-Orbea, et al. 1999. BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. *J. Exp. Med.* 189:1747.
45. Sugai, S., D. W. Palmer, N. Talal, and I. P. Witz. 1974. Protective and cellular immune responses to idiotype determinants on cells from a spontaneous lymphoma of NZB-NZW F<sub>1</sub> mice. *J. Exp. Med.* 140:1547.
46. Daley, M. J., H. M. Gebel, and R. G. Lynch. 1978. Idiotype-specific transplantation resistance to MOPC-315: abrogation by post-immunization thymectomy. *J. Immunol.* 120:1620.
47. Kwak, L. W., R. Pennington, L. Boni, A. C. Ochoa, R. J. Robb, and M. C. Popescu. 1998. Liposomal formulation of a self lymphoma antigen induces potent protective antitumor immunity. *J. Immunol.* 160:3637.
48. King, C. A., M. B. Spellerberg, D. Zhu, J. Rice, S. S. Sahota, A. R. Thompson, T. J. Hamblin, J. Radl, and F. K. Stevenson. 1998. DNA vaccines with single-chain Fv fused to fragment C of tetanus toxin induce protective immunity against lymphoma and myeloma. *Nat. Med.* 4:1281.
49. Koch, F., U. Stanzl, P. Jennewein, K. Janke, C. Heufler, E. Kampgen, N. Romani, and G. Schuler. 1996. High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. *J. Exp. Med.* 184:741.
50. Wynn, T. A., A. Reynolds, S. James, A. W. Cheever, P. Caspar, S. Hieny, D. Jankovic, M. Strand, and A. Sher. 1996. IL-12 enhances vaccine-induced immunity to schistosomes by augmenting both humoral and cell-mediated immune responses against the parasite. *J. Immunol.* 157:4068.
51. Kaminski, M. S., K. Kitamura, D. G. Maloney, M. J. Campbell, and R. Levy. 1986. Importance of antibody isotype in monoclonal anti-idiotype therapy of a murine B cell lymphoma: a study of hybridoma class switch variants. *J. Immunol.* 136:1123.
52. Davis, T. A., D. G. Maloney, D. K. Czerwinski, T. M. Liles, and R. Levy. 1998. Anti-idiotype antibodies can induce long-term complete remissions in non-Hodgkin's lymphoma without eradicating the malignant clone. *Blood* 92:1184.